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Full Length Research Paper

Morphology and histochemistry of the glandular trichomes of *Isodon rubescens* (Hemsley) H. Hara [Lamiaceae]: A promising medicinal plant of China

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***Isodon rubescens*, a perennial herb indigenous to China, with medicinal application, potentially has economic value. The morphology of the glandular trichomes was investigated with light microscopy. At the same time the chemical content was analyzed by applying chemical reagents and fluorescence microscopy. This morphoanatomical and histochemical study revealed that leaves of *I. rubescens* possess one type of non-glandular and two types of glandular trichomes, with the latter differing both anatomically and in the composition of their secondary metabolites. Non-glandular trichomes were uniseriate with an ornamented surface. Peltate and capitate glandular trichomes comprised one basal cell, one stalk cell and one head. The head of mature peltate glandular trichomes consisted of four-twelve secretory cells while that of the capitate glandular hairs was comprised of two cells. Peltate glandular trichomes containing compounds of terpenoid nature are probably the main site of oridonin and ponicidin accumulation. The fluorescent stain of peltate and capitate glandular trichomes indicated the possible presence of phenolic compounds.**

Key words: *Isodon rubescens*, Lamiaceae, glandular trichomes, microscopy, histochemistry, terpenoids, phenolic.

INTRODUCTION

The genus *Isodon* (Schrad. ex Benth.) Spach (Lamiaceae) contains ca. 100 species distributed predominantly in tropical and subtropical Asia, with the center of diversity in southwestern China with outliers in tropical Africa (Wu and Li 1977; Codd 1984; Li 1988; Li and Hedge, 1994). The use of *Isodon* species in Chinese popular folk medicine has a long tradition, among which, *Isodon rubescens* (Hemsley) H. Hara, a perennial herb of the *Isodon* genus, is native to the Yellow River valley of China. The leaves of *I. rubescens*, which is the most studied species and is known in China by the name, "*donglingcao*", are still used by the local people in Henan province for the treatment of respiratory and gastro-intestinal bacterial infections, inflammation, and cancer (Sun et al., 2006). Most Lamiaceae species have glandular trichomes that emerge from the epidermal surface of their leaves, stems and reproductive structures. Generally, trichomes are divided into two subcategories, glandular and non-glandular (Wagner et al., 2004). Glandular trichomes secrete various types of compounds. A growing body of experimental evidence shows that terpene biosynthesis takes place within these trichomes (Croteau and Johnson, 1984; Hay and Svoboda, 1993; Duke et al., 2000; Hallahan, 2000; Siebert, 2004). Terpenes usually constitute the major lipophilic components of these secretions**.** Pharmacological study has shown that the major constituents of *I. rubescens* are diterpenoids, especially oridonin and ponicidin, which have significant antiangiogenic activity (Meade-Tollin et al., 2004).

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Other types of compounds found in *I. rubescens* include triterpenoids and flavonids. However, to date, knowledge of the morphology and functioning of *I. rubescens* trichomes has been absent, therefore, we carried out an investigation of the morphology, distribution, and histochemistry of the glandular trichomes present on leaves of *I. rubescens*, with the aim of clarifying their role. The investigations described here are motivated by an interest in the pharmacological properties of the species. The identification of structures responsible for oridonin and ponicidin production and accumulation is useful from an economic standpoint because it can be used to develop strategies for maximizing yields of useful compounds. This information is also meaningful from an ecological perspective because it can help us better understand what role these compounds may have in the plant's ecology, and may lead to a better understanding of natural plant protection.

MATERIALS AND METHODS

Plant material

Fresh aerial plant parts of *I. rubescens* were harvested in August (2010) from the Chinese Herb Garden, Henan College of Traditional Chinese Medicine. For the purpose of these investigations, the term 'mature leaves' refers to vegetative leaves harvested from the fourth and fifth nodes of the plant, whilst 'young leaves' describes leaves that had recently emerged from the first node. Light microscopy and histochemical investigations were performed on fresh, as well as air-dried material.

Light microscopy

Leaf material was cut into small pieces (5 mm^2) in the laboratory and fixed in FAA (formalin–glacial acetic acid–70% ethanol, 5: 5 : 90, v/v) for 48 h. The sample was dehydrated in an ethanol dilution series (30 50, 70, 85, 95 and 100 % ethanol, followed by 2 × 100% xylene). The sample was then embedded in wax before sectioning to 10 to 15 µm thick using a microtome dissector. The sections were dewaxed using two changes of xylene, and then rehydrated using descending grades of ethanol (up to 50%) and finally water. The Sections were stained with 0.5% toluidine blue-O for approximately 2 min, and briefly dehydrated in ascending grades of alcohol. The alcohol was washed off (2 washes) in xylene before mounting using neutral balsam. Slides were viewed and photographed with a Leica DM3000 light microscope. Leaf clearing was done with 10% NaOH solution followed by water and further clearing with 25% Sodium hypochlorite. Chloral hydrate 250% solution was used as mordant, specimens were mounted on slides with neutral balsam and photographed with a Leica DM3000 light microscope.

Histochemical investigations

A Natural Product reagent was prepared by mixing aqueous AlCl3 solution 5 and 0.05% diphenylboric acid-b-ethylaminoester in 10% methanol as described by Heinrich et al. (2002) for the detection of flavonoids. Entire leaves were soaked in the solution for 10 min, after which the plant material was dried on absorbent paper and mounted on a glass slide. Vanillin-HCl was prepared by dissolving 0.1 g vanillin in 100 ml concentrated HCl (Nikolakaki and Christodoulakis, 2004). This reagent is a universal indicator of various compounds, enabling differentiation between different classes of secondary metabolites such as flavonoids, terpenoids and phenyl carboxylic acids based on the colouration obtained (Wagner and Bladt, 1996). Leaves were soaked for 2 min and then dried on absorbent paper before being mounted on a glass slide. Hand-cut sections of fresh leaf tissue were stained with vanillin–HCl for terpenoids. Slides were viewed directly under UV and GF (Green fluorescence) light with a Leica DM5500 fluorescence microscope.

RESULTS

The indumentum of *I. rubescens* leaves consisted of glandular and non-glandular trichomes distributed on both the adaxial and abaxial surfaces (Figure 1A and B). The non-glandular trichomes were distributed mainly on the veins and leaf margins and appeared more abundant on the abaxial leaf surface. In young leaves, the non-glandular trichomes partially obscured the glandular trichomes and it appeared that they matured at an early stage of leaf development (Figure 1A). With leaf expansion, both non-glandular and glandular trichomes density decreased progressively as shown in Figure 1. Morphologically, non-glandular trichomes were uniseriate, and sharply pointed with warts on the surface (Figure 1C). The glandular trichomes were classified into two types: peltate and capitate. (Figure 1B and D). The peltate glandular trichomes consisted of a basal cell embedded in the epidermis, a unicellular stalk and a large spherical or slightly flattened multicellular secretory head (Figure 1 I). The heads of mature peltate glandular trichomes generally consisted of four cells that were enclosed in a smooth cuticle, but twelve-celled heads were also found (Figure 1E and F). The head cells were grouped together above the stalk cell, surrounding its central axis (Figure 1E). The fully developed head was quadrilobate and 40 ± 2 µm in diameter. The elevated cuticle of the head possibly accumulated secretory material (Figure 1 I). The secretions caused the cuticle to lift and expand, giving it a tumescent, somewhat globular appearance (Figure 1D and F). The peltate glandular trichomes had a single basal cell, a unicellular stalk and a bicellular secretory head (Figure 1G and H) The capitate glandular trichomes were typically 18 ± 2 µm tall, with a head that is 18 ± 2 µm in diameter. Peltate and capitate glandular trichomes were also observed on the stems, cotyledons, rachises, pedicles, bracts and calyces.

Histochemistry of glandular trichomes

On the young leaf surface, numerous capitate glandular trichomes had a blue fluorescence (Figure 2A and 2B) under UV light when stained with the Natural Product reagent, indicating the presence of flavonoids. Peltate

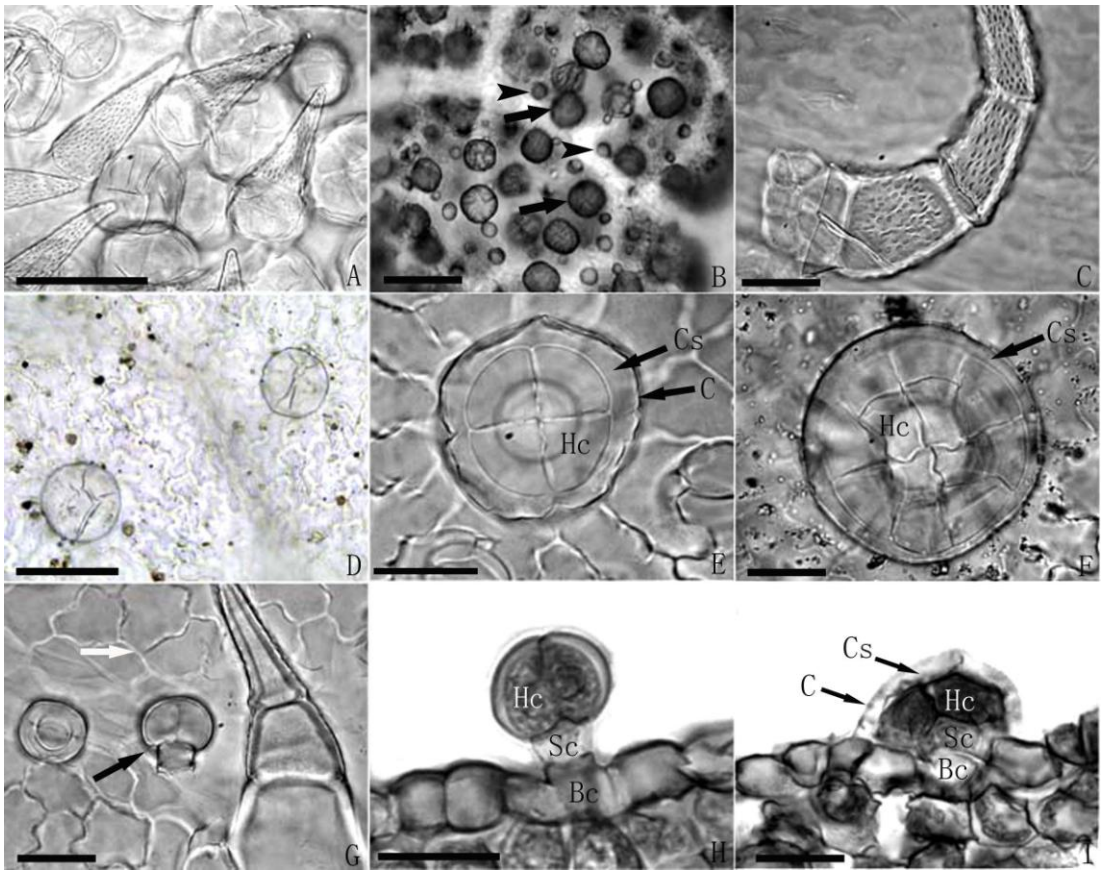

Figure 1. Trichomes of *I. rubescens*. A. Glandular and non-glandular trichomes on the abaxial leaf surface of a young leaf. Bar = 50 µm. B. Capitate (arrowheads) and peltate (arrows) glandular trichomes on the abaxial leaf surface. Bar = 100 µm. C. Non-glandular trichome with warts on leaf surface. Bar = 50 µm. D. Mature peltate glandular trichome. Bar = 50 µm. E. Mature peltate glandular trichome with 4-celled secretory head (H) assembly. The circle in the center indicates the anticlinal wall of the stalk cell. Arrows indicate cuticle(C) and subcuticular space (Cs). Bar = 20 µm. F. Mature peltate glandular trichome with 12-celled secretory head (H) assembly. The arrow indicates subcuticular space (Cs). Bar = 20 µm. G. The arrow indicates capitate glandular trichome on leaf surface. Bar = 50 µm. H. Capitate glandular trichome in longitudinal section. Bar = 20 µm. I. Peltate glandular trichome in longitudinal section. Arrows indicate cuticle(C) and subcuticular space (Cs). Bar = 20 µm. (H and I). Bc = basal cell; Hc = head cell; Sc = stalk cell.

glandular trichomes of mature leaves showed intense fluorescence after being treated with the natural product reagent (Figure 2D and 2E) or vanillin–HCl (Figure 2F, 2G and 2H), respectively. After 10 days of drying, peltate glandular trichomes still showed intense fluorescence following staining with vanillin–HCl (Figure 2I). However, the rest of the tissue did not fluoresce following staining with vanillin–HCl (Figure 2I). Capitate glandular trichomes didn't fluoresce on mature or dried leaves as shown in Figure 2.

DISCUSSION

Like other members of the Lamiaceae, *I. rubescens* leaves possess one type of non-glandular and two types of glandular trichomes (called peltate and capitate), on both adaxial and abaxial surfaces. The density of trichomes, including non-glandular and glandular, gradually decreases with leaf maturity. The observation that non-glandular trichomes did not give any positive reaction supports the argument that their role is protection from water loss, the regulation of temperature through their reflective capacity and mechanical protection from herbivores (Ascensão et al., 1995, 1999; Yashodhara et al., 2001).The density of glandular trichomes decreases with leaf growth (young leaves have a denser pubescence). This might be an adaptive mechanism, whereby the young leaves, most tender and appetizing to herbivores, are given the highest protection (many secretions of glandular trichomes are deterrent or toxic to insects). The density of glandular trichomes is further considered to be associated with transpiration, leaf overheating, UV-B radiation, etc. (Wagner et al.,

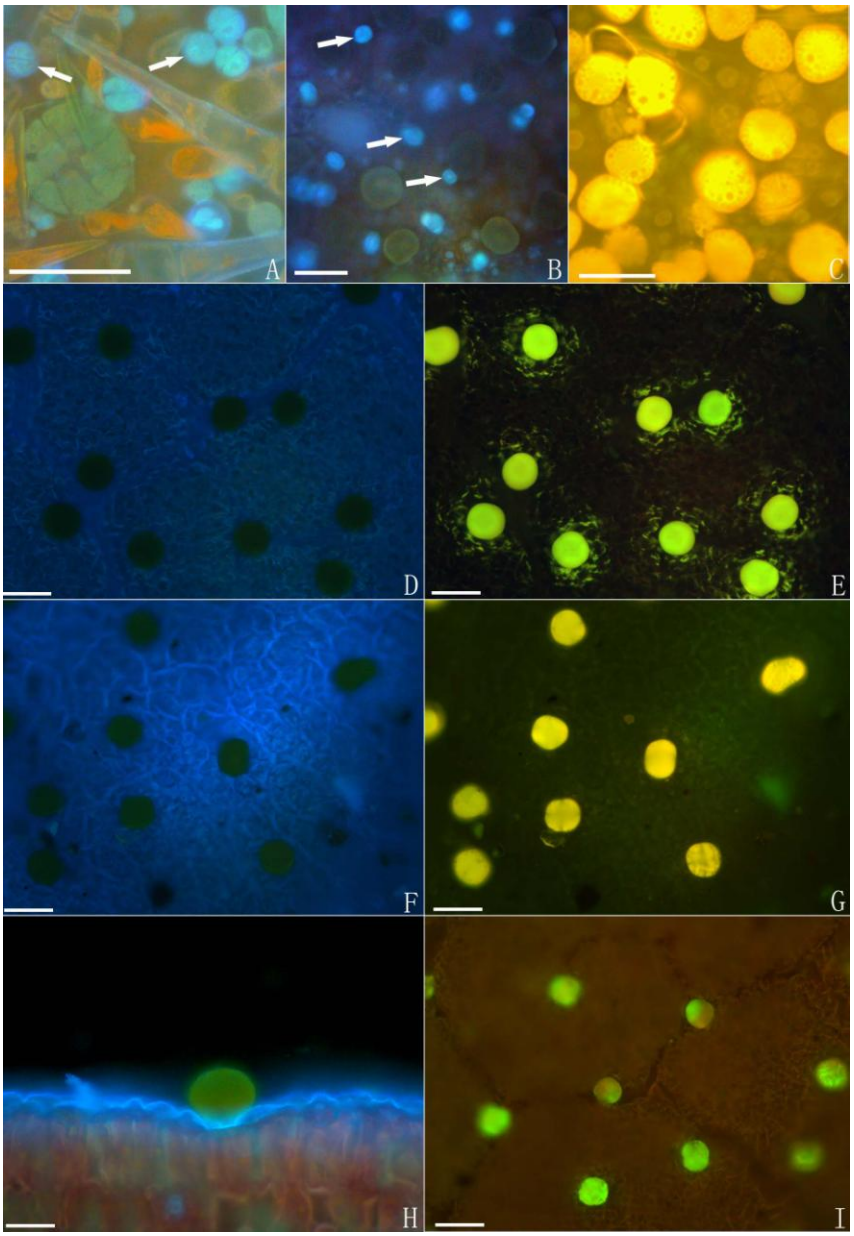

Figure 2A and B. Arrows indicate capitate glandular trichomes of a young leaf following staining with the Natural Product reagent. C. Peltate glandular trichomes on the adaxial surface of a young leaf following staining with vanillin–HCl. D and E. Paired micrographs of peltate glandular trichomes on a mature leaf surface fluoresce after staining with the Natural Product reagent. F and G. Paired micrographs of peltate glandular trichomes on a mature leaf surface after staining with vanillin–HCl. H. Fluorescence micrograph of free-hand section using mature leaves. Note the bright fluorescence of peltate trichome after staining with vanillin–HCl. I. Peltate glandular trichomes on a dried leaf surface fluoresce after staining with vanillin–HCl. A, B, D, F and H. Indicate glandular trichomes that exhibit fluorescence when exposed to UV light, while C, E, G and I. indicate fluorescence when exposed to green fluorescence (GF) light. Scale bars: H = 100 µm, others = 50 µm.

2004). Most studies on glandular trichomes use histochemical methods (Ascensão et al., 1999; Bisio et al., 1999; Corsi and Bottega 1999; Bottega and Corsi 2000; Nikolakaki and Christodoulakis 2004, 2007) because they are considered useful for an initial investigation of the presence of some substances. In this

work, histochemical tests are applied for the first time to the leaves of *I. rubescens*. Autofluorescence is diagnostic of flavonoids, which, depending on the structural type, show dark yellow, green or blue fluorescence under UV light (Wagner and Bladt, 1996). Natural product reagent was used to enhance the natural autofluorescence of phenolic compounds (Andary et al., 1984). The fact that capitate glandular trichomes exhibited light blue fluorescence when GF was used indicated presence of flavonoids (Figure 2B). However, in mature leaves capitate glandular trichomes showed no fluorescence. Observations made during this study suggest that the content of capitate glandular trichomes are metabolically altered during leaf maturation and result in disappearance of fluorescence. These short-term capitate glandular trichomes are assumed to function for a very short period during the early development of young organs (Fahn, 1988; Duke and Paul, 1993). As compared to the capitate glandular trichomes, peltate glandular trichomes of *I. rubescens* have a short one-celled stalk and a large round or slightly flattened head. During the secretion phase, peltate glandular trichomes of *I. rubescens* have a characteristic spherically shaped head due to the development of a large sub-cuticular space where possibly secretory products accumulate. Terpenoid secretion was found to be restricted to peltate glandular trichomes. The remarkable colour, typifying terpenoids, obtained with vanillin–HCl (Figure 2F and 2G) was due to the mixed colour reactions of individual terpenoids. However, this reaction is not specific for diterpenoids. So, the reaction does not necessarily indicate that the peltate glandular trichomes contain diterpenoids. More telling is the lack of a reaction in the rest of the tissue. The vanillin–HCl is quite sensitive to terpenoids, so a lack of a reaction is a clear indication that diterpenoids are absent in these tissues. The presence of phenolic compounds in peltate trichomes was also indicated by colour reactions. The fact that peltate trichomes exhibited yellow fluorescence when GF was used indicated presence of flavonoids. The different colour reactions between peltate trichomes and capitate trichomes using the natural product reagent indicating structural difference of flavonoids. The peltate glandular trichomes of *I. rubescens* are regarded as long-term glandular trichomes in which the secretory material appears to accumulate gradually and consistently under elevated cuticular sacs during the development and growth of the aerial parts of *I. rubescens*. These glandular trichomes are believed to play a vital role in defensive mechanisms against the pathogens and herbivores (Werker, 1993)**.** Present observations suggest that oridonin and ponicidin are possibly only present in peltate glandular trichomes. The number of glandular trichomes on the leaves is linearly associated with the yield in terpenes. Thus, the greater the number of glandular trichomes on the leaves, the higher the amount of terpene substances derived from them by distillation (Bosabalidis and Kokkini, 1997). This is due to the fact that the glandular trichomes are the main leaf sites of terpene biosynthesis and possess a complete enzymatic equipment (McCaskill and Croteau, 1995). Oridonin yields from leaves are up to 20 times as high as from stems (Lu et al., 2000). This dramatic differentiation is primarily due to the fact that the stems are largely composed of woody vascular tissue and pith, which do not contain oridonin. Stems typically weigh about ten times as much as leaves when dry sections of both organs with equal adaxial surface areas are compared. The density of peltate glandular trichomes on the abaxial stem surface is about the same as that on the abaxial leaf surface (Siebert, 2004). The concentration of these compounds in whole stems and whole leaves differs in a manner that roughly corresponds to their different ratios of weight to abaxial surface area. Oridonin yield is directly related to the distribution and density of terpenoid-bearing glands. In the case of *I. rubenscens*, whole plants would have to be utilized for any industrial production of oridonin since stems are difficult to separate from leaves. However our study does not provide direct proof that these compounds are present inside, or are derived from peltate glandular trichomes. Only further investigation (for example, through shearing off trichomes, isolating them and analyzing their content) will determine which type of trichomes is responsible for each of the various compounds.

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